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(54) Title: IMPROVED IMMUNE COMPLEX ASSAY

(57) Abstract

An improved method for detecting disease-related antigen in a patient sample is provided. Antigen which is present in an immune complex is detected by exposing a patient sample to a solid phase having immobilized binding substance for the immune complexes. The immune complexes are thus captured and may subsequently be separated from the sample by removing the solid phase. The presence of the disease-related antigen within the separated immune complex can then be detected on the solid phase by conventional means.

IMPROVED IMMUNE COMPLEX ASSAY

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to immunoassays for the detection of disease-related antigens. More particularly, the present invention relates to immunoassays wherein the detected antigen is present in an immune complex in a patient sample.

In the field of immunology, there have been numerous reports relating to the identification of disease-associated antigens, often referred to as "disease markers." Such antigens may be circulating plasma components, intracellular molecules, or cell surface molecules. Typically, these are proteins, glycoproteins, glycolipids, and the like, which are associated with normal or abnormal plasma components and/or with abnormal cells but not with normal cells or with normal cells in very small quantities. Many attempts have been made to correlate the serum or plasma level of such antigens with the presence and/or status of the disease.

Unfortunately, such correlations have been very difficult to make. Ideally, a useful marker would be present in all patients suffering from a particular disease but would be absent from the sera or plasma of all normal subjects and patients suffering from other types of diseases. At present, no such markers have been identified. Generally, it has been found that markers associated with a particular disease may not be universally associated with that disease in all patients and may sometimes be found in normal subjects. Such assays are thus subject to "false" positives.

For these reasons, it would be desirable to provide an improved marker assay capable of furnishing a

superior correlation between the assay maker and the disease in question. It would be particularly desirable to provide such assays which may be employed with both known and presently undiscovered markers.

The detection of tumor-associated antigen in immune complexes has been proposed. In one such protocol, immune complexes are detected by exposure to immobilized Raji cells which bind to the immune complexes. In another protocol, it has been proposed to first enrich immune complexes by passage of patient plasma through a protein A or similar column. The complexes are then separated by elution from the column, and presence of antigen in the enriched fraction is effected. Such previously-proposed protocols, however, suffer from certain disadvantages.

The use of Raji cells for immune complex detection suffers from several disadvantages. There is a need for continuous maintenance of the cell line in tissue culture. With continuous passage of the cell line, there is the possibility of changes within the cell line which will in turn affect the immune complex binding characteristics of the cells. There is also the potential for the cell line to die out; therefore, several early passages of the cell line must be cryogenically preserved as backup sources for the cell line. In addition, there are the general inherent problems associated with tissue culture techniques that must be considered, such as the potential for bacterial or viral contamination. Moreover, caution must also be exercised to ensure that samples analyzed do not contain antibodies reactive with the Raji cells, thus leading to false positive results in the assay.

The use of solid phase protein A as an initial enrichment step suffers some disadvantages. Although protein A is an excellent entity for selectively enriching immune complexes, the disassociation of these immune complexes from protein A requires somewhat harsh

conditions which include dramatic pH changes or treatment with chaotropic agents. After such treatment, not all of the immune complexes may reassociate for further analyses and, indeed, some immune complexes may be irreversibly damaged. Thus, it would be desirable to bind and further analyze the immune complexes in a single step without the need to dissociate complexes formed in an initial extraction step.

2. Description of the Background Art

U.S. Patent No. 4,711,839, describes an immune complex assay including an enrichment step, typically passage of patient plasma through an affinity column specific for the immune complexes. The enriched complexes are then separated from the column eluant, and the separated complexes screened for tumor-associated antigen. Gupta et al. (1983) Clin. Exp. Immunol. 53:589-599, describe the detection of tumor-associated antigen in eluates from *Staphylococcus aureas* columns used for the immunoadsorption of plasma from a melanoma patient. Theofilopoulos et al. (1978) J. Clin. Invest. 59:1570-1581, describe an assay for immune complexes employing Raji cells for binding of the immune complexes. The relationship between immune complexes and human disease is described in detail in Theofilopoulos and Dixon (1980) Am. J. Path. 531-594.

SUMMARY OF THE INVENTION

The present invention provides methods for diagnosing and monitoring antigen-related diseases, such as autoimmune disorders or cancers, by detecting disease-related antigens which are present as part of circulating immune complexes in a patient sample. The measurement of antigens found in such immune complexes has been found to provide an improved correlation with disease status when compared to methods which measure free disease-related antigen, either with or without detection of immune

complexes. Preferred methods of the present invention will measure complex-associated disease-related antigen to the exclusion of free disease-related antigen.

In a first aspect of the present invention, an aliquot of a patient sample, such as blood, plasma, serum, urine, or the like, is exposed to a solid phase having immobilized receptor for immune complexes, such as protein A, C1q, C3b receptor, anti-Ig antibody, anti-C1q antibody, anti-C3b antibody, or the like. By then separating the solid phase from the sample, substantially all immune complexes initially present in the sample are retained by the solid phase to the exclusion of any free antibody which may have been present in the sample. Immune complexes which contain the disease-related antigen are then detected on the separated solid phase.

In a second aspect of the present invention, antigen related to an autoimmune disorder is detected in a patient sample by first introducing an aliquot of the sample into a test well coated with receptor specific for immune complexes. The receptor is present in an amount greater than that needed to bind all immune complexes which could reasonably be expected to be present in the aliquot of the patient sample. By then removing the liquid sample from the test well, substantially all immune complexes initially present in the sample remain bound to the test well. An antibody which binds specifically to the autoimmune antigen is then introduced to the test well, also in an amount greater than that needed to bind all autoimmune antigen which could reasonably be expected to be in the test well. Binding between the antibody and the autoimmune antigen is then detected in the test well.

In a third and preferred aspect of the present invention, a tumor antigen or "marker" is detected in the sample using substantially the same method steps described for detecting autoimmune antigens.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Serum samples from normal subjects and autoimmune thrombocytopenia (ITP) patients were assayed for the presence and levels of platelet antigen component gpIIbIIIa in circulatory immune complexes (CIC). Note the elevated levels of antibody specific for gpIIbIIIa in serum samples from ITP patients as compared with serum samples from normal subjects. Moreover, the mean value for the ITP group was significantly (P=0.0286) elevated as determined statistically by the Mann-Whitney two sample test.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Antigen and antibody may form freely in plasma to form circulatory immune complexes (CIC). Normally, the presence of antigen in circulation is sufficiently low, or the antigen is present for sufficiently brief time, that the CIC are removed by the phagocytic mechanisms of the immune system. However, because of disease activity, high concentrations of disease antigen may persist for long periods of time. It is believed that the formation of these complexes may have two effects which relate to the detection of disease-associated antigen in serum or plasma. First, because the antigen is involved in such complexes, the antigen may be refractory to detection by normal immunoassays. This situation would lead to false negative results. Second, because low levels of antigen may be present in normal individuals which will not form CIC, false positive results can be detected in normal immunoassays. Both sources of error may be obviated by detecting disease associated antigen only as present in CIC, as accomplished by the method of the present invention.

The assay of the present invention comprises an immune complex capture step followed by a disease-related antigen detection step. The capture step utilizes a solid phase substrate which has an immune complex binding

substance immobilized on a surface thereof. The immune complex binding substance will be present in an excess amount, i.e., an amount more than sufficient to bind all immune complexes which might reasonably be expected to be present in the patient sample. By then separating the solid phase from the patient sample and washing the solid phase surface to remove non-bound proteins and other substances, the immune complexes initially present in the sample will remain bound to the solid phase in the absence of any free antigen which may have been present. The presence of the disease-related antigen in the bound immune complexes may then be detected on the separated solid phase surface.

Suitable patient samples include any patient specimen which is a liquid or may be liquified and which might include immune complexes containing the antigen of interest. Preferred patient samples include blood, serum, plasma, lymph fluid, and the like. Other suitable patient samples include urine, saliva, tears, and the like. Particularly preferred is the use of patient serum samples.

Liquid patient samples will be divided into aliquots having a volume suitable for the particular assay protocol, as described in more detail below. Suitable aliquot volumes will be in the range from about 0.5 ml to about 5.0 ml, usually being in the range from about 1 ml to 3 ml. Usually, it will be unnecessary to pretreat the sample prior to testing, although in some cases filtration to remove particulate contaminants will be employed. Serum and plasma, of course, will be obtained from whole blood by conventional separation techniques.

The antigen to be detected will be characteristic of a particular disease to be diagnosed or monitored. The antigen will elicit a humoral immune response in the patient which results in the formation of circulating immune complexes, as described generally

above. Of particular interest to the present invention are antigens which elicit an immune response and which are thus responsible for an immune-related disorder such as autoimmune disease or neoplastic disease (cancer). Such antigens include platelet antigen component gpIIbIIIa, which is characteristic of immune thrombocytopenia, Le^x glycolipid antigen, which is characteristic of adenocarcinoma, rheumatoid factor, which is characteristic of rheumatoid arthritis, glutamic acid decarboxylase, which is characteristic of Type 2 diabetes, and the like. Preferred tumor markers for detection by the method of the present invention include carcinoembryogenic antigen (CEA); prostate-specific antigen (PSA); CA 125 (ovarian cancer); CA 15-3 (breast cancer); CA 19-9 (pancreatic cancer); neuron-specific enolase (NSE; tumors of neuroendocrine origin); mammary serum antigen (MSA); mucinous-like carcinoma-associated antigen (MCA); and tumor-associated glycoprotein (TAG) 72.4. Antibodies and other natural binding receptors capable of specifically recognizing each of these antigens are well described in the patent and scientific literature.

The present invention will employ a solid phase substrate which may be contacted with the patient sample of interest and thereafter separated from said patient sample while remaining physically intact. Preferably, this solid phase will be composed of a relatively rigid, non-swellable material which is dimensionally stable before, during, and after contact with the patient sample. A variety of suitable materials are available, including glass; ceramics; and plastics, such as polypropylene, polystyrene, polyvinyl chloride, polyethylene terephthalate, solid phase polymeric resins, and the like.

Suitable immune complex binding substances are well known in the art and include protein A, C1q complement protein, C3b receptor protein, anti-human

immunoglobulin (anti-Ig) antibody, anti-C1q antibody, anti-C3b antibody, and the like. These binding substances are all proteins and may be immobilized on the solid phase substrate by a variety of conventional techniques. Such techniques typically employ heterobifunctional linking reagents, and may optionally require the derivatization of either or both of the protein and substrate surface prior to binding. Such binding techniques are well described in the patent and scientific literature. See, for example, Voller et al., in *Enzyme-Linked Immunosorbent Assay*, second ed., Rose and Friedman, eds., 1980, pp. 359-371, the full disclosure of which is incorporated herein by reference.

Presence of the disease-related antigen in the immune complex will be detected using an antibody or natural binding receptor or ligand for the antigen. The introduction of an observable label to the antigen will be mediated by the introduction of the antibody, receptor, or ligand. Most directly, the antibody for the disease-related antigen may itself be coupled to the observable label so that binding between the antibody and the antigen will result in introduction of the label to the solid phase. A variety of other indirect binding techniques for introduction of the label to the solid phase are also available. For example, an unlabeled primary antibody may be introduced to the solid phase which binds to the antigen of interest. The label may then be introduced using a labeled secondary antibody which is specific for the primary antibody, e.g., anti-(mouse antibody) antibody as the secondary antibody when the primary antibody is mouse antibody. In a preferred alternative, a biotinylated antibody specific for the antigen may first be introduced to the solid phase, followed by the introduction of labeled avidin. The very high binding affinity between biotin and avidin (including streptavidin) enhances the introduction of label using this latter technique. Such biotin-avidin

bridge labelling techniques are described in Hsu et al. (1981) Am. J. Clin. Pathol. 75:734, the disclosure of which is incorporated herein by reference.

A variety of detectable labels are suitable for use in the present invention. Enzymes are of particular interest, including hydrolases, particularly esterases and glycosidases, and oxidoreductases, particularly peroxidases. Also suitable are radioactive labels, such as ^{125}I, ^{32}P, ^{14}C, and the like; fluorescent compounds such as fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, and the like; and chemiluminescers such as luciferin and luminol, and the like. These detection labels may be used in solid or liquid phase systems.

Assays according to the present invention will be run to capture and isolate a sufficient amount of immune complex from the patient sample to permit subsequent detection of disease-related antigen in the immune complex without the need to dissociate the antigen from its bound antibody in the complex. This is done by providing excess binding substance specific for the immune complex on a solid phase surface to contact the patient sample and capture substantially all immune complex which would be reasonably expected to be present in the tested volume of sample. The solid phase is then removed from the sample, and the sample surface washed so that the presence of disease-related antigen on the solid phase surface may be detected. By detecting only the disease-related antigen present in immune complexes, an improved correlation with disease status has been found.

An exemplary assay will employ microtiter plates, each including a plurality of test wells, as the solid phase. The immune complex capture substance, typically anti-C1q or anti-C3 antibody, is immobilized on the inner surface of each test well, and aliquots of liquid patient sample are then introduced to each test well to effect capture of the immune complexes by the

immune complex binding substances. The patient sample may then be withdrawn from the test wells, and each test well then washed to remove unbound substances. Antibody or other binding substance specific for the antigen-related disease is next introduced to the test well, and binding between the binding substances and antigen may be detected using conventional techniques, typically those utilized in enzyme-linked immunosorbent assay (ELISA) systems. For example, enzyme-labeled antibodies specific for the antigen may be introduced in a suitable buffer. The remaining reagents necessary to produce a colored, fluorescent, or luminescent signal are then introduced to the wells to provide a detectable signal which is qualitatively and/or quantitatively indicative of the amount of antigen-related antigen in the immune complexes in the patient sample.

EXPERIMENTAL

Materials and Methods

Specific antibodies to complement protein component C1q were affinity purified by passage of goat antiserum over an immunoadsorbent column containing covalently bound purified human complement component C1q. The affinity purified antibodies were concentrated by precipitation with 50% ammonium sulfate after which they were dissolved in 0.01 M phosphate buffered saline (PBS), pH 8.6, and extensively dialyzed against PBS, pH 8.6. Microtiter wells were then coated with 10ug of affinity purified anti-C1q antibody overnight at 25°C. These microtiter wells could then be employed as a solid phase substrate for specifically binding C1q containing CIC present in serum or plasma samples.

For the disease marker assay, we utilized serum samples from normal subjects and from patients diagnosed with immune thrombocytopenia (ITP). ITP is an immune disorder in which patients develop antibodies against their own platelets and are at risk for a fatal bleeding

episode due to abnormally low amounts of circulating platelets which are required for adequate coagulation of a bleeding event.

In this assay, serum samples were diluted 1/10 in PBS, pH 7.5, containing 1% bovine serum albumin (BSA). The samples were then incubated in microtiter wells for 60 minutes at 25°C. After incubation, the wells were washed 3 times with PBS, pH 7.5, containing 1% polysorbate (PBS/polysorbate). After washing, the wells were incubated with a solution containing biotin labeled monoclonal antibody to platelet antigen component gpIIbIIIa for 60 minutes at 25°C. After incubation, the wells were washed 3 times with PBS/polysorbate. After washing, the wells were incubated with a solution containing peroxidase conjugated Strep-avidin for 30 minutes at 25°C. After incubation, the wells were washed 3 times with PBS/polysorbate. After washing, substrate solution containing o-phenylenediamine with H_2O_2 as a catalyst was added to each well and incubated at 25°C. After appropriate color development, the reaction was stopped by addition of 5 N sulfuric acid. The absorbance was measured in an ELISA plate reader at 492nm.

Results

In order to determine the disease specificity of the ITP related CIC, the assay of the present invention was performed on 4 normal subjects and 4 patients diagnosed with ITP. As shown in Fig. 1, significantly higher levels of labeled monoclonal antibody specific for the disease antigen were detected in patient samples indicating the presence of disease specific platelet antigen present in CIC. This indicates that enrichment of CIC on solid phase substrates will allow for the detection of disease specific antigen present in the immune complexes.

Although the foregoing invention has been described in some detail by way of illustration and

example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the stated claims.

WHAT IS CLAIMED IS:

1. A method for detecting a disease-related antigen in a patient sample, said method comprising:

exposing an aliquot of the sample to a solid phase having immobilized binding substance for immune complexes;

separating the solid phase and the sample, whereby immune complexes initially present in the sample remain bound to the solid phase; and

detecting the presence of immune complexes which contain the disease-related antigen on the separated solid phase.

2. A method as in claim 1, wherein the solid phase is selected from the group consisting of microtiter wells, test tubes, dip sticks, and beads.

3. A method as in claim 2, wherein the immune complex receptor is selected from the group consisting of protein A, C1q, C3b receptor, anti-Ig antibody, anti-C1q antibody, and anti-C3b antibody.

4. A method as in claim 3, wherein immune complexes are detected on the solid phase by (a) exposing the solid phase to a labeled substance which binds specifically to the antigen and (b) observing the presence of label on the solid phase.

5. A method as in claim 3, wherein immune complexes are detected on the solid phase by (a) exposing the solid phase to an intermediate substance which binds specifically to the binding sites on the intermediate substance, and (b) observing the presence of label on the solid phase.

6. A method as in claim 5, wherein the intermediate binding substance is a biotinylated antibody which binds to the antigen and the labeled substance is labeled avidin.

7. A method as in claim 4, wherein the labeled substance includes a label selected from the group consisting of enzymes, radioactive substances, fluorescent substances, chemiluminescent substances, and bioluminescent substances.

8. A method as in claim 1, wherein the disease-related antigen is a tumor antigen.

9. A method for detecting an antigen related to an immune-related or a neoplastic disorder in a patient sample, said method comprising:

introducing an aliquot of the patient sample to a test well coated with receptor specific for immune complexes, wherein the amount of coated receptor is greater than that needed to bind all immune complex which is expected to be in the aliquot of patient sample;

removing the liquid sample from the test well, whereby substantially all immune complexes initially present in the sample remain bound to the test well;

introducing to the test well antibody which binds specifically to the immune-related antigen in an amount needed to bind all antigen greater than that expected to be in the aliquot of patient sample; and

detecting binding between the antibody and the autoimmune antigen in the test well.

10. A method as in claim 9, wherein the immune complex receptor is selected from the group consisting of protein A, C1q, C3b receptor, anti-Ig antibody, anti-C1q antibody, and anti-C3b antibody.

11. A method as in claim 10, wherein the antibody introduced to the test well is attached to a detectable label, and binding between the antibody and the immune-related antigen is detected by observing the presence of label bound to the test well.

12. A method as in claim 10, wherein the antibody introduced to the test well is biotinylated, and binding between the antibody and the immune-related antigen is detected by introducing labeled avidin and observing the presence of label bound to the test well.

13. A method as in claim 11, wherein the label is selected from the group consisting of enzymes, radioactive substances, fluorescent substances, chemiluminescent substances, and bioluminescent substances.

14. A method as in claim 12, wherein the label is selected from the group consisting of enzymes, radioactive substances, fluorescent substances, chemiluminescent substances, and bioluminescent substances.

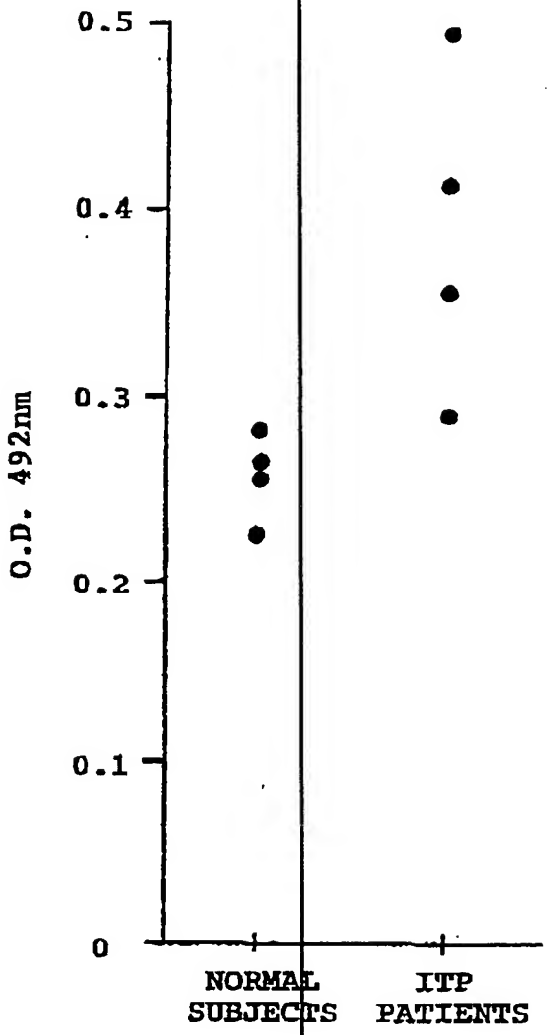

Figure 1